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## Hyperandrogenemia Predicts Metabolic Phenotype in Polycystic Ovary Syndrome: The Utility of Serum Androstenedione

Michael W. O'Reilly, Angela E. Taylor, Nicola J. Crabtree, Beverly A. Hughes, Farfia Capper, Rachel K. Crowley, Paul M. Stewart, Jeremy W. Tomlinson,\* and Wiebke Arlt\*<sup>†</sup>

**Context:** Polycystic ovary syndrome (PCOS) is a triad of anovulation, insulin resistance, and hyperandrogenism. Androgen excess may correlate with metabolic risk and PCOS consensus criteria define androgen excess on the basis of serum T. Here we studied the utility of the androgen precursor serum androstenedione (A) in conjunction with serum T for predicting metabolic dysfunction in PCOS.

**Patients and Methods:** Eighty-six PCOS patients fulfilling Rotterdam diagnostic consensus criteria and 43 age- and body mass index-matched controls underwent measurement of serum androgens by tandem mass spectrometry and an oral glucose tolerance test with homeostatic model assessment of insulin resistance and insulin sensitivity index calculation. We analyzed 24-hour urine androgen excretion by gas chromatography/mass spectrometry.

**Results:** PCOS patients had higher levels of serum androgens and urinary androgen metabolites than controls (all  $P < .001$ ). Within the PCOS cohort, both serum A and T were positively correlated with the free androgen index ( $T \times 100/\text{SHBG}$ ) and total androgen metabolite excretion (all  $P < .001$ ). All subjects with T above the normal reference range [high T (HT)] also had high A (HA/HT group,  $n = 56$ ). However, the remaining 30 patients had normal T levels, either in the presence of HA (HA/NT;  $n = 20$ ) or normal A (NA/NT;  $n = 10$ ). The groups did not differ in age or BMI. The HA/HT and HA/NT groups had higher total androgen excretion than NA/NT ( $P < .01$  and  $P < .05$ , respectively). Multiple linear regression showed a strong negative association between serum androstenedione and insulin sensitivity. The incidence of dysglycemia according to an oral glucose tolerance test increased with the severity of androgen phenotype (NA/NT, 0%; HA/NT, 14%; HA/HT, 25%,  $P = .03$ ).

**Conclusion:** Simultaneous measurement of serum T and A represents a useful tool for predicting metabolic risk in PCOS women. HA levels are a sensitive indicator of PCOS-related androgen excess. (*J Clin Endocrinol Metab* 99: 1027–1036, 2014)

\* J.W.T. and W.A. contributed equally to this work.

<sup>†</sup> Author affiliations are shown at the bottom of the next page.

Abbreviations: A, androstenedione; AE+Anov, androgen excess and anovulation; AE+Anov+PCO, androgen excess, anovulation, and polycystic ovaries on ultrasound; An, androsterone; Anov+PCO, anovulation and polycystic ovaries on ultrasound; BMI, body mass index; CI, confidence interval; DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate; DXA, dual-energy x-ray absorptiometry; Et, etiocholanolone; FAI, free androgen index; GC/MS, gas chromatography/mass spectrometry; HA/HT, high A and also T above the normal reference range; HA/NT, high A and normal T levels; HOMA-IR, homeostasis model assessment of insulin resistance; 11 $\beta$ -HSD1, 11 $\beta$ -hydroxysteroid dehydrogenase type 1; 17 $\beta$ -HSD5, 17 $\beta$ -hydroxysteroid dehydrogenase type 5; ISI, insulin sensitivity index; LC/MS-MS, liquid chromatography/tandem mass spectrometry; NA/HT, normal A and T above the normal reference range; NA/NT, normal A and normal T levels; OGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; THE, tetrahydrocortisone; 5 $\alpha$ -THF/THF, 5 $\alpha$ -tetrahydrocortisol to tetrahydrocortisol; 5 $\alpha$ -THF + THF, tetrahydrometabolites of cortisol.

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**P**olycystic ovary syndrome (PCOS) is defined by a clinical triad of anovulation, insulin resistance, and androgen excess (1). It has an estimated prevalence of 5%–10% in women of reproductive age and consumes most assisted conception health care budgets (2, 3). Diagnostic criteria for PCOS have changed significantly in recent years, acknowledging the heterogeneity of clinical phenotype (4, 5). The long-term health consequences of PCOS are increasingly recognized, ranging from metabolic dysfunction to increased cardiovascular events (6–8). Androgen excess remains the cardinal clinical feature that defines the condition and may contribute at least in part to the adverse metabolic phenotype.

However, a lack of clarity persists on which androgens should be measured, what constitutes normal reference ranges, and which analytical technique should be used (9). Equally we have little insight into whether different patterns of biochemical hyperandrogenemia define distinct PCOS subgroups in terms of metabolic risk. The most accurate strategy for quantification of biochemical androgen excess in PCOS is the measurement of total 24-hour urinary androgen metabolite excretion (10); however, urinary steroid profiling by gas chromatography/mass spectrometry (GC/MS) is time consuming and expensive. In routine clinical practice, serum total T remains the most commonly measured and widely available marker for the estimation of biochemical androgen excess (2). However, the measurement of T is severely limited by a number of factors including the following: 1) T circulates bound to SHBG and other proteins such as albumin, and only the unbound or free fraction enters into target tissues (11); 2) the measurement of free T by direct RIA is highly inaccurate (12); 3) direct immunoassays for total T are highly variable (13); and 4) T circulates in low nanomolar range concentrations, with the lower end of the normal female reference range below the limit of quantitation in all assays available.

Androgen excess in PCOS originates from the ovaries and adrenals (14); moreover, increased peripheral conversion of T to the most potent androgen 5 $\alpha$ -dihydrotestosterone by systemically up-regulated 5 $\alpha$ -reductase activity has been well documented (15, 16). Androstenedione (A) is the immediate precursor of T. In addition to direct secretion by ovaries and adrenals, A can be generated in peripheral tissues from its precursor dehydroepiandrosterone (DHEA) by the enzyme 3 $\beta$ -hydroxysteroid dehydrogenase type 2 (17). In addition to mainly ovarian production, about 25% of plasma T derives from the

conversion from A by 17 $\beta$ -hydroxysteroid dehydrogenase type 5 (17 $\beta$ -HSD5 or AKR1C3) in adipose and other peripheral tissues (18). Serum A is inconsistently measured in routine clinical investigation of suspected PCOS because clinicians remain uncertain about its diagnostic value. Limited previous work suggests that 10% of PCOS patients may be misclassified as normoandrogenemic if A is not measured (19).

The independent health risks of androgen excess in women are increasingly apparent (20). We therefore hypothesized that patients with the coelevation of A and T and therefore increased circulating androgen burden represent a distinct subgroup with adverse metabolic parameters compared with those with normal androgens or solitary elevation of either A or T. We thus also hypothesized that A represents a more sensitive marker for the biochemical detection of androgen excess. We therefore carried out a detailed analysis of androgen synthesis and metabolism to test these hypotheses in a large cohort of women with PCOS according to Rotterdam criteria in comparison with a group of body mass index (BMI)-matched healthy controls.

## Materials and Methods

### Subjects

Women with PCOS were recruited from outpatient clinics at the Queen Elizabeth Hospital Birmingham and Birmingham Women's Hospital. This observational, cross-sectional study was approved by the South Birmingham Research Ethics Committee, and all participants gave their written informed consent. PCOS was diagnosed according to the Rotterdam European Society of Human Reproduction and Embryology 2004 criteria, with the presence of two or more of the following: oligo-/anovulation, clinical signs of hyperandrogenism, and polycystic ovaries on ultrasound (4). Other causes of oligomenorrhea and/or androgen excess were excluded by history, physical examination, and biochemical assessment. Healthy controls were recruited via local advertisement, with a diagnosis of PCOS excluded by clinical and biochemical parameters. Exclusion criteria for both groups were current or recent treatment with glucocorticoids, congenital adrenal hyperplasia, hyperprolactinemia, thyroid dysfunction, pregnancy, age younger than 18 or older than 45 years, oral contraceptive use within 3 months prior to recruitment, and diabetes mellitus.

### Clinical protocol

Participants attended the National Institute of Health Research/Wellcome Trust Clinical Research Facility at the Queen Elizabeth Hospital Birmingham after an overnight fast. Each

patient provided a 24-hour urine sample for urinary steroid metabolite analysis by GC/MS as previously reported (21). Baseline anthropometric assessment included height, weight, BMI (kilograms per square meter), and blood pressure measurement. A modified Ferriman-Gallwey scoring system for hirsutism was used as described previously (22). Dual-energy x-ray absorptiometry (DXA) was used for body composition assessment using a Hologic Discovery/W DXA (software version Apex 3.0; Hologic Inc). Specific fat phenotype was measured using android, gynecoid, peripheral (arms and legs), and trunk regions of interest, with subsequent calculation of android to gynecoid and trunk to peripheral fat ratios (23).

Blood samples were drawn for fasting plasma glucose and insulin and serum T, A, DHEA, DHEA sulfate (DHEAS), SHBG, FSH and LH, total cholesterol, high-density lipoprotein, and triglycerides. Each participant underwent a 75-g oral glucose tolerance test (OGTT) with blood sampling at 30-minute intervals for 2 hours. Glucose and insulin were measured using methods described previously (24). Subjects were categorized as having normal glucose tolerance or dysglycemia on the basis of 2-hour glucose values (normal glucose tolerance <7.8 mmol/L, dysglycemia  $\geq 7.8$  mmol/L). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: [fasting glucose (millimoles per liter) \* fasting insulin (milliinternational units per liter)/22.5]. Insulin sensitivity index (ISI) was calculated using the following formula:  $ISI = [75\,000 + (\text{glucose}_0 - \text{glucose}_{120}) \times 1.15 \times 180 \times 0.19 \times \text{body weight (kg)}] / [120 \times \log(\text{insulin}_{\text{mean}} \times \text{glucose}_{\text{mean}})] = (\text{mg} \cdot \text{l}^{-2} \cdot \text{mmol}^{-1} \cdot \text{mIU}^{-1} \cdot \text{min}^{-1})$  (25, 26).

### Serum and urine steroid measurements

Serum steroids were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS) using a Waters Xevo mass spectrometer with Acquity uPLC system. LC/MS-MS conditions were an electrospray ionization source with capillary voltage 4.0 kV, a source temperature of 150°C, and a desolvation temperature of 500°C. Serum steroid oxime analysis, which facilitates enhanced detection by formation of oxime derivatives of the steroid oxogroups (27), was used for the measurement of T, A, and DHEA and carried out in positive mode, whereas the measurement of serum DHEAS was performed in negative mode. T, A, and DHEA were extracted from 200  $\mu\text{L}$  serum via liquid-liquid extraction using 1 mL tert-butyl-methyl-ether followed by derivatization into steroid oximes using 100  $\mu\text{L}$  derivatization mixture (0.16 g hydroxylamine in 8 mL pyridine). For protein precipitation and extraction of DHEAS, 20  $\mu\text{L}$   $\text{ZnSO}_4$ , 0.1 mM, and 100  $\mu\text{L}$  acetonitrile were added to 20  $\mu\text{L}$  serum before evaporation under constant nitrogen flow (28). All steroids were separated using an optimized gradient system consisting of methanol with 0.1% formic acid and quantified referring to a linear calibration series with appropriate internal standards. Each steroid was identified by matching retention times and two mass transitions in comparison with a deuterated reference compound.

Serum A and T were categorized as normal based on locally derived LC/MS-MS reference values for premenopausal women (T, 0.3–1.9 nmol/L; A, 1.4–7.4 nmol/L; percentiles of 2.5th to 97.5th of an unselected healthy female control cohort). Serum androgens were used to categorize PCOS patients into four subgroups: normal A/normal T (NA/NT), high A/normal T (HA/NT), normal A/high T (NA/HT), and high A/high T (HA/HT). The free androgen index (FAI) was calculated using the formula

$(T \times 100)/\text{SHBG}$ . We also subdivided the PCOS patients into the four phenotypic subgroups arising from the Rotterdam criteria (29) as recommended at a recent National Institutes of Health meeting (3). These subgroups are defined by the presence of the following: 1) androgen excess, anovulation, and polycystic ovaries on ultrasound (AE+Anov+PCO), 2) Anov+PCO, 3) AE+PCO and 4) AE+Anov.

Urinary steroid metabolite excretion analysis was carried out by GC/MS as described previously (21). The sum of androstosterone (An) and etiocholanolone (Et) was considered representative of active androgen metabolite excretion (10). Net systemic  $5\alpha$ -reductase activity was assessed by the ratios of An to Et and  $5\alpha$ -tetrahydrocortisol to tetrahydrocortisol ( $5\alpha$ -THF/THF).  $11\beta$ -hydroxysteroid dehydrogenase type 1 ( $11\beta$ -HSD1) activity was estimated using the ratio of the tetrahydrometabolites of cortisol ( $5\alpha$ -THF + THF) to tetrahydrocortisone (THE) (30), and  $11\beta$ -hydroxysteroid dehydrogenase type 2 ( $11\beta$ -HSD2) activity by the ratio of free urinary cortisol to cortisone. Total glucocorticoid metabolite excretion was assessed as the sum of  $5\alpha$ -THF + THF + THE + cortolones + cortols + cortisol + cortisone.

### Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 21 was used for data analysis. All data are expressed as mean  $\pm$  SD unless otherwise stated. Independent-samples *t* tests or Mann-Whitney *U* tests were used as appropriate for comparison between two groups. One-way ANOVA with post hoc Tukey testing was used for multiple comparisons between different groups. Correlation testing was performed using Pearson's correlation coefficient or Spearman's test as appropriate. Multiple linear regression was used to adjust for the confounding effects of different variables. Differences were considered statistically significant at  $P < .05$ .

## Results

Baseline clinical and metabolic characteristics of PCOS and age- and BMI-matched healthy controls are shown in Table 1. Complete clinical, biochemical, and radiological data were available in 86 women with PCOS. Results were compared with those obtained from 43 age- and BMI-matched controls. Hirsutism, oligomenorrhea, and polycystic ovaries were present in 87%, 92%, and 73% of PCOS patients, respectively. Polycystic ovary subgroups arising from the Rotterdam criteria were matched for age and BMI and categorized as follows: 1) AE+Anov+PCO ( $n = 51$ , 59%); 2) AE+PCO ( $n = 6$ , 7%); 3) Anov+PCO ( $n = 9$ , 11%); and 4) AE+Anov ( $n = 20$ , 23%).

### Circulating androgens and androgen excretion

Both serum A and T were significantly higher in PCOS than in healthy controls ( $P < .0001$  for both, Table 1). PCOS patients also had significantly higher FAI, DHEA, and DHEAS (all  $P < .0001$ ) than controls. Total 24-hour urinary androgen excretion was also significantly higher



**Table 1.** Clinical, Biochemical, and Metabolic Characteristics in the Overall PCOS Cohort (n = 86), PCOS Androgen Phenotype Subgroups, and Age- and BMI-Matched Controls (n = 43)

Variable	Controls (n = 43)	All PCOS (n = 86)	NA/NT (n = 10)	HA/NT (n = 20)	HA/HT (n = 56)
Age, y	32.4 ± 9.8	30.0 ± 7.2	32.9 ± 2.9	30.8 ± 7.9	28.8 ± 7.0
BMI, kg/m <sup>2</sup>	30.3 ± 6.4	31.9 ± 7.1	30.2 ± 6.5	30.0 ± 5.9	32.1 ± 6.5
Ferriman-Gallwey score	2.1 ± 1.6	12.4 ± 6.7 <sup>a</sup>	9.9 ± 5.3 <sup>a</sup>	10.1 ± 6.4 <sup>a</sup>	13.9 ± 7.2 <sup>a,b</sup>
T, nmol/L	1.3 ± 0.4	2.2 ± 0.8 <sup>a</sup>	1.2 ± 0.3	1.6 ± 0.4	2.6 ± 0.7 <sup>a,b</sup>
FAI (T × 100/SHBG)	3.2 ± 1.8	9.1 ± 6.1 <sup>a</sup>	5.5 ± 3.9 <sup>d</sup>	7.3 ± 4.6 <sup>d</sup>	10.5 ± 6.5 <sup>a,b</sup>
SHBG, nmol/L	49.8 ± 26.6	32.3 ± 18.4 <sup>a</sup>	28.8 ± 11.8 <sup>a</sup>	30.8 ± 21.2 <sup>a</sup>	31.8 ± 16.5 <sup>a</sup>
A, nmol/L	3.5 ± 1.4	13.4 ± 5.6 <sup>a</sup>	5.1 ± 1.7	11.4 ± 4.2 <sup>a,c</sup>	14.2 ± 5.1 <sup>a,c</sup>
DHEA, nmol/L	20.5 ± 7.9	57.5 ± 30.0 <sup>a</sup>	24.4 ± 8.6	59.8 ± 34.8 <sup>a,e</sup>	58.0 ± 26.8 <sup>a,e</sup>
DHEAS, μmol/L	3.9 ± 2.0	6.2 ± 3.0 <sup>a</sup>	2.4 ± 1.2	6.3 ± 2.6 <sup>c</sup>	6.2 ± 2.7 <sup>a,c</sup>
DHEA to DHEAS ratio	7.8 ± 3.7	12.9 ± 6.8 <sup>d</sup>	12.6 ± 8.5 <sup>d</sup>	14.0 ± 10.0 <sup>d</sup>	15.4 ± 5.4 <sup>d</sup>
LH, IU/L	5.2 ± 2.4	9.9 ± 5.8 <sup>d</sup>	4.7 ± 3.2	9.0 ± 6.3 <sup>d</sup>	11.4 ± 9.2 <sup>b,d</sup>
LH to FSH ratio	0.8 ± 0.5	2.0 ± 1.5 <sup>d</sup>	0.9 ± 0.7	2.1 ± 1.9 <sup>d</sup>	2.3 ± 1.4 <sup>b,d</sup>
Fasting glucose, mmol/L	4.6 ± 0.5	4.8 ± 0.7	5.0 ± 0.5	4.7 ± 0.5	4.8 ± 0.8
Two-hour OGTT glucose, mmol/L	6.7 ± 1.5	6.5 ± 2.2	5.5 ± 1.2	6.7 ± 1.9	6.7 ± 2.3
Fasting insulin, mIU/L	2.0 ± 1.5	10.4 ± 8.6 <sup>f</sup>	8.2 ± 5.5 <sup>d</sup>	11.7 ± 8.3 <sup>d</sup>	12.3 ± 6.6 <sup>a</sup>
HOMA-IR	0.9 ± 0.8	2.3 ± 0.2 <sup>f</sup>	1.9 ± 1.1 <sup>d</sup>	2.4 ± 1.5 <sup>d</sup>	2.8 ± 1.0 <sup>b,f</sup>
ISI (Cederholm), mg/L <sup>2</sup> /mmol · mU · min (25)	78.7 ± 38.0	54.6 ± 24.1 <sup>a</sup>	73.4 ± 54.6	51.0 ± 17.1 <sup>a,f</sup>	54.4 ± 25.2 <sup>a,f</sup>
Dysglycemia, n, %	3 (6.9%)	17 (19.7%) <sup>d</sup>	0	3 (15%)	14 (25%) <sup>b,d</sup>

Abbreviation: HOMA-IR, homeostatic model assessment of insulin sensitivity. PCOS subgroup classification according to high (H) or normal (N) serum A and serum T concentrations as measured by LC/MS-MS [normal T (NT) ≤1.9 nmol/L; normal A (NA) ≤7.4 nmol/L]. Data are expressed as mean ± SD unless otherwise stated. Normal glucose tolerance was a 2-hour glucose OGTT 7.7 mmol/L or less. Dysglycemia was a 2-h glucose OGTT 7.8 mmol/L or greater.

<sup>a</sup> *P* < .001 as compared with the BMI-matched healthy controls.

<sup>b</sup> *P* < .05 as compared with the NA/NT group.

<sup>c</sup> *P* < .001 as compared with the NA/NT group.

<sup>d</sup> *P* < .05 as compared with the BMI-matched healthy controls.

<sup>e</sup> *P* < .01 as compared with the NA/NT group.

<sup>f</sup> *P* < .01 as compared with the BMI-matched healthy controls.

in PCOS (*P* < .0001, Table 2), with higher excretion of An (*P* < .001), Et (*P* < .05), and DHEA (*P* < .05). T and A correlated strongly with FAI and total androgen metabolite excretion (Figure 1, A–D).

Serum A was elevated in 76 of 86 PCOS patients (88.3%); T was elevated in 56 (65.1%). A and T were elevated in 1 of 43 (2.3%) and 5 of 43 (11.6%) healthy controls, respectively. Serum levels of A and T were in the normal range in 10 and 30 PCOS patients, respectively. Patients with PCOS were subsequently divided into four androgen phenotype groups dependent on circulating androgens; NA/NT, NA/HT, HA/NT, and the most severe degree of androgen excess, HA/HT. Most patients were categorized as HA/HT (n = 56), with 20 and 10 patients classified as HA/NT and NA/NT, respectively; no patient had high T but normal A (Figure 2).

Serum androgen levels in the PCOS women with the mildest degree of biochemical androgen excess (NA/NT) were similar to those in healthy BMI-matched controls (Table 1). However, they differed significantly from controls with regard to SHBG and FAI. NA/NT women had androgen excretion rates similar to controls, whereas an-

drogen excretion in the two other PCOS groups was significantly higher (Table 2 and Figure 3A). Similarly, serum DHEA and DHEAS were significantly higher in HA/HT and HA/NT patients as compared with the NA/NT group and the controls (Figure 3, E and F). The Ferriman-Gallwey score was significantly higher in HA/HT women compared with NA/NT (*P* = .04) (Table 1).

### Metabolic characteristics

Overall, PCOS patients had higher fasting insulin and HOMA-IR values than BMI-matched controls, but fasting glucose and 2-hour values did not differ significantly (Table 1). The incidence of dysglycemia was 19.7% in the PCOS group compared with 6.9% in controls (*P* = .03) (Table 1). BMI was positively correlated with FAI (*R* = 0.4, *P* < .0001) and systemic 5α-reductase activity (An/Et, *R* = 0.21, *P* = .01; 5α-THF + THF, *R* = 0.20, *P* = .02). ISI values were lower in PCOS patients compared with controls (*P* < .001, Table 1). The ISI had a strong negative correlation with serum T and A (*R* = −0.41, *P* < .001, and *R* = −0.55, *P* < .00001, respectively, Figure 1, E and F). When PCOS and control patients were considered sepa-

**Table 2.** Twenty-Four-Hour Urinary Steroid Excretion in PCOS Patients, Controls, and PCOS Androgen Phenotype Subgroups

Variable	Controls (n = 43)	All PCOS (n = 86)	NA/NT (n = 10)	HA/NT (n = 20)	HA/HT (n = 56)
Total androgen metabolites, μg per 24 h	2882 ± 2018	5493 ± 2781 <sup>a</sup>	2751 ± 1760	4886 ± 1968 <sup>b,c</sup>	5727 ± 2530 <sup>a,c</sup>
An, μg per 24 h	1413 ± 906	2824 ± 1651 <sup>a</sup>	1452 ± 1178	2594 ± 1207 <sup>b</sup>	3016 ± 1553 <sup>a,c</sup>
Et, μg per 24 h	1468 ± 1208	2457 ± 725 <sup>a</sup>	1299 ± 733	2292 ± 1211	2683 ± 1469 <sup>b,d</sup>
An/Et ratio (5α-reductase activity)	1.1 ± 0.4	1.3 ± 0.6 <sup>b</sup>	1.2 ± 0.6	1.3 ± 0.6	1.3 ± 0.6
Urinary DHEA, μg per 24 h	457 ± 721	1740 ± 954 <sup>b</sup>	477 ± 400	1586 ± 960	1960 ± 854 <sup>b,c</sup>
Total glucocorticoid metabolites, μg per 24 h	6821 ± 3572	9624 ± 4214 <sup>e</sup>	8449 ± 4181	8935 ± 3404	9803 ± 4390 <sup>b</sup>
5α-THF, μg per 24 h	940 ± 608	1313 ± 814 <sup>b</sup>	1250 ± 1135	1259 ± 759	1359 ± 842 <sup>e</sup>
THF, μg per 24 h	1206 ± 607	1520 ± 549 <sup>b</sup>	1489 ± 924	1477 ± 475	1634 ± 822 <sup>b</sup>
5α-THF/THF (5α-reductase activity)	0.8 ± 0.3	0.9 ± 0.5	0.8 ± 0.4	0.8 ± 0.5	0.9 ± 0.5
(5α-THF+THF)/THE (11β-HSD1 activity)	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.4
Urinary cortisol/urinary cortisone (11β-HSD2 activity)	0.6 ± 0.5	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.2

Abbreviations: total androgen metabolites, sum of An+Et; total glucocorticoid metabolites, sum of THF+5α-THF+THE+cortols+cortolones+free cortisol/cortisone. Androgen and glucocorticoid metabolites were measured by GC/MS. The PCOS subgroup classification was according to high (H) or normal (N) A and T concentrations as measured by LC/MS-MS [normal T (NT) ≤1.9 nmol/L; normal A (NA) ≤7.4 nmol/L]. Data are expressed as mean ± SD unless otherwise stated.

<sup>a</sup>  $P < .001$  as compared with the BMI-matched healthy controls

<sup>b</sup>  $P < .05$  as compared with the BMI-matched healthy controls.

<sup>c</sup>  $P < .05$  as compared with the NA/NT group.

<sup>d</sup>  $P < .01$  as compared with the NA/NT group.

<sup>e</sup>  $P < .01$  as compared with the BMI-matched healthy controls.

ately, a negative association between A and ISI was observed in both groups ( $R = -0.53$ ,  $P < .00001$ , and  $R = -0.41$ ,  $P < .001$ , respectively). In obese control patients, this negative association between A and ISI persists ( $R = -0.25$ ,  $P = .04$ ). On multiple linear regression analysis, a significant negative association was confirmed only between A and ISI [coefficient  $-2.69$ , 95% confidence interval (CI)  $-1.57$  to  $-3.81$ ,  $P < .0001$ , Supplemental Table 1, published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>]; no such association was observed between ISI and serum T, DHEAS, or DHEA.

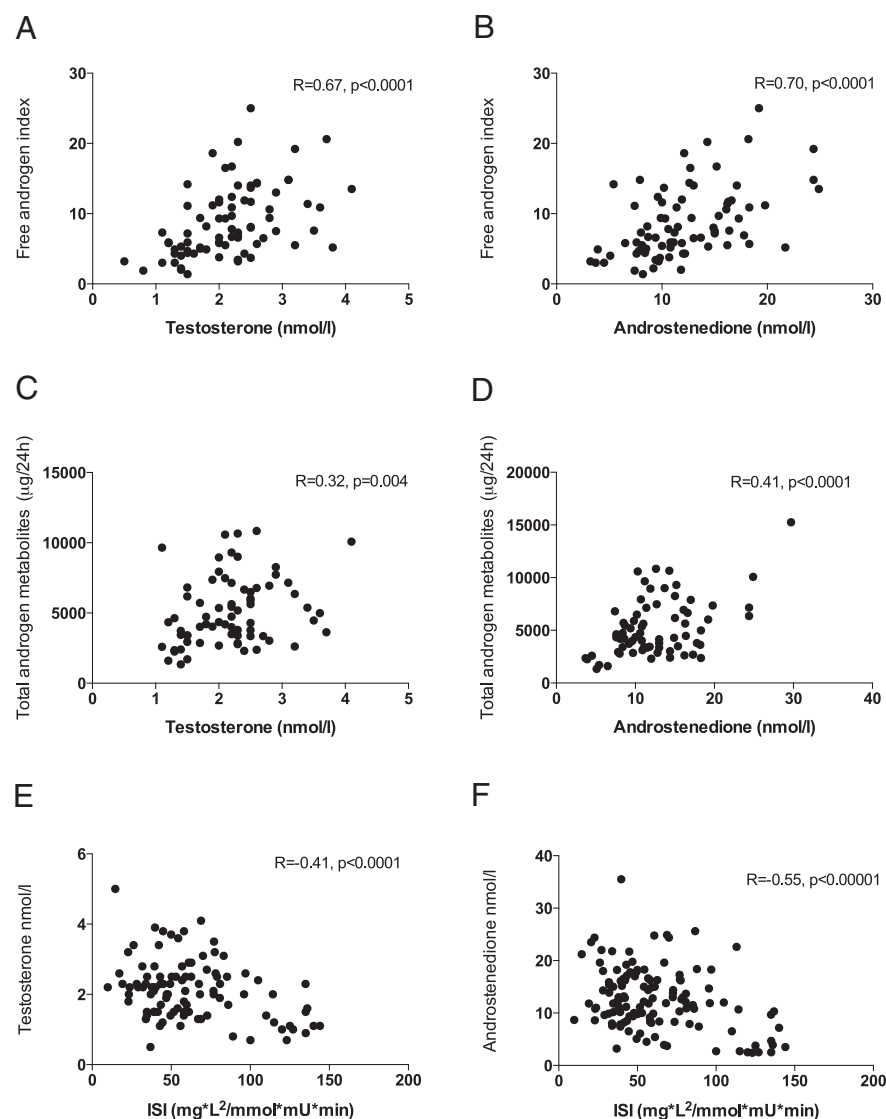
HOMA-IR in all three PCOS subgroups was significantly higher than in controls and HA/HT women had significantly higher values than NA/NT women ( $P < .05$  for both) (Figure 3B). However, only HA/NT and HA/HT had lower ISI values (Table 1). PCOS women in the NA/NT group had significantly higher fasting insulin and HOMA-IR than BMI-matched controls (Table 1). The rate of dysglycemia in the HA/NT and HA/HT groups was 15% and 25%, respectively; no NA/NT women were dysglycemic. No significant differences were observed in systolic blood pressure or lipid profiles between each group. No difference in insulin sensitivity was observed between the four phenotypic groups defined by the Rotterdam criteria (Supplemental Figure 1).

## Body composition

No significant differences in fat distribution or total lean mass on DXA were observed between PCOS and control women or between the three androgen phenotype or Rotterdam phenotype groups (see Supplemental Table 2). The android to gynecoid ratio was strongly correlated with HOMA-IR ( $R = 0.53$ ,  $P < .0001$ ), FAI ( $R = 0.37$ ,  $P < .0001$ ), An/Et ( $R = 0.38$ ,  $P < .0001$ ), 5α-THF/THF ( $R = 0.31$ ,  $P < .0001$ ) and total glucocorticoid metabolites ( $R = 0.26$ ,  $P = .01$ ). There was a strong negative correlation between the android to gynecoid ratio and ISI ( $R = -0.41$ ,  $P < .01$ ).

## Cortisol metabolism

PCOS patients and controls did not differ significantly in indices of 11β-HSD1 or 11β-HSD2 activity (Table 2). However, total glucocorticoid excretion was higher in the PCOS group ( $9624 \pm 4214$  vs  $6821 \pm 3572$  μg per 24 h,  $P = .01$ ). Urinary glucocorticoid excretion appeared to gradually increase with the degree of androgen excess, with significantly higher levels in the HA/HT group than in controls (Table 2). On multiple linear regression (Supplemental Table 1), total glucocorticoid excretion was a negative predictor of ISI (coefficient  $-0.003$ , 95% CI  $-0.001$  to  $-0.005$ ,  $P = .048$ ).



**Figure 1.** A–F, Relationship of serum T and A with FAI, total urinary androgen metabolites, and ISI. There is a significant positive relationship between both T and A with total androgen excretion and the FAI; a negative association with ISI is observed.

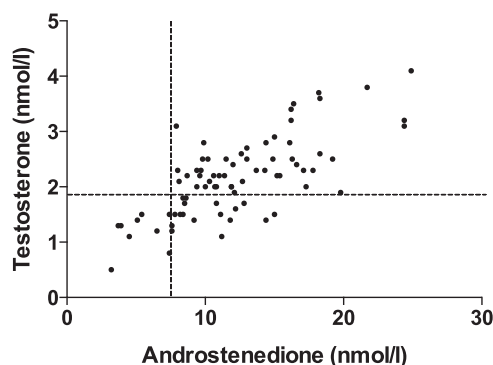
## Discussion

We have performed a detailed assessment of the clinical, metabolic and androgen phenotype of a cohort of PCOS, revealing that PCOS patients with coelevation of A and T have impaired indices of insulin sensitivity compared with those with normal androgens or milder hyperandrogenemia. Using multiple linear regression, we found a strong negative relationship between A and insulin sensitivity as measured by the ISI, independent of age and BMI; such a relationship was not observed for T. All our patients were diagnosed according to consensus criteria, fulfilling at least two of three criteria. Until relatively recently, it has been unclear whether women with hyperandrogenic PCOS are at increased cardiometabolic risk compared with those without androgen excess. However, recent data suggest that biochemical an-

drogen excess poses a higher risk of liver disease, insulin resistance, and subclinical atherosclerosis compared with those with anovulation and polycystic ovaries alone (20, 31, 32). These studies suggest that hyperandrogenic PCOS represents a distinct metabolic phenotype, whereas patients without androgen excess may have a phenotype similar to healthy controls (33). However, in our cohort we also found higher fasting insulin values in normoandrogenemic PCOS compared with healthy controls, suggesting that women without androgen excess are not entirely protected from metabolic dysfunction.

Women with hyperandrogenemic PCOS in our cohort had a significantly reduced ISI; those with normal serum androgens had ISI values comparable with healthy controls. It is interesting that HOMA-IR and homeostatic model assessment of insulin sensitivity values were still unfavorable in the NA/NT group, suggestive perhaps of impaired fasting indices but preserved ability to handle an oral glucose load. Further studies are needed to corroborate and understand the associations observed here. Serum androgens, and in particular A, had a strong negative association with the ISI; it is interesting that this association was also observed in control patients with circulating A levels within the normal range. These data lend support to the hypothesis that metabolic disease in PCOS should be considered as a continuous variable, with metabolic dysfunction observed across a worsening spectrum of severity of androgen excess. It is important to remember that normal reference ranges are relatively arbitrarily defined and that androgen exposure across all patients may contribute to their metabolic phenotype. Women with normal circulating androgens may still exhibit subtle hyperandrogenemia after LH stimulation (34), which could explain the higher indices of insulin resistance, lower SHBG, and higher FAI levels observed. The long-term implications of this are unclear without longitudinal, prospective studies, but these findings have also been observed by Barber et al (31). When we subdivided our PCOS cohort into the four phenotypic groups arising from the

drogen excess poses a higher risk of liver disease, insulin resistance, and subclinical atherosclerosis compared with those with anovulation and polycystic ovaries alone (20, 31, 32). These studies suggest that hyperandrogenic PCOS represents a distinct metabolic phenotype, whereas patients without androgen excess may have a phenotype similar to healthy controls (33). However, in our cohort we also found higher fasting insulin values in normoandrogenemic PCOS compared with healthy controls, suggesting that women without androgen excess are not entirely protected from metabolic dysfunction.



**Figure 2.** Relationship of serum T and A with stratification of androgenemic subgroups: NA/NT ( $n = 10$ ); HA/NT ( $n = 20$ ); and HA/HT ( $n = 56$ ). No individuals were identified with NA/HT ( $n = 0$ ).

Rotterdam consensus (Supplemental Figure 1), no significant differences were observed in insulin sensitivity or body composition between each group. It seems reasonable to argue that this strategy of subphenotyping of PCOS patients may not accurately predict those at highest metabolic risk. Admittedly, the AE+Anov+PCO group comprised most patients, but a similar distribution of PCOS patients was also noted by Barber et al (31). Interestingly, Barber et al found that patients in their AE+Anov+PCO subgroup were more insulin resistant; however, the groups were not BMI matched, unlike in our study. For this reason we believe that comeasurement of A and T might offer a better alternative in terms of metabolic risk prediction than the previously suggested categorization by phenotype.

It is crucial that PCOS patients are correctly and accurately categorized as normoandrogenemic or hyperandrogenemic if they are to be stratified into low- and high-risk metabolic groups on this basis. Our data show that the measurement of serum T alone fails to adequately make this differentiation. Elaborate investigative tools such as LH stimulation testing or 24-hour urinary androgen metabolite excretion are not universally available in routine clinical practice. By contrast, concurrent measurement of serum T and A provides a suitable alternative. In our view, only those with normal serum levels of both androgens should be categorized as normoandrogenemic; as discussed above, even this cohort may still exhibit occult hyperandrogenism, and without prospective studies it is impossible to be certain whether they are protected from more severe metabolic disease over time. To date, there are only very limited data on the effects of androgen-lowering therapies on metabolic parameters in PCOS (35, 36), and clearly larger prospective trials are needed.

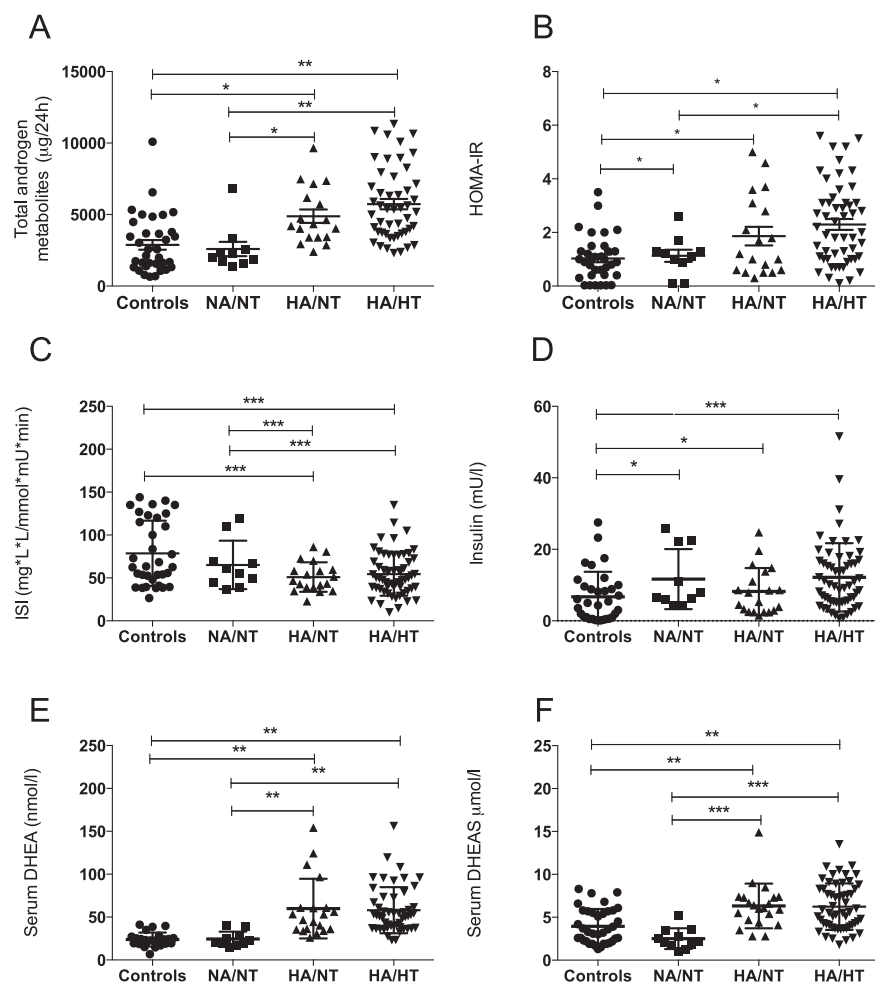
Only 65% of our patients had elevated serum T, whereas 88% had serum A concentrations above the reference range. In addition, more than two thirds of the women initially categorized as normoandrogenemic on the basis of a normal T had androgen excess as docu-

mented by increased serum A levels, revealing that the proportion of patients with biochemical androgen excess is significantly larger than previously reported (19). Based on the supposition that none of our controls have occult PCOS, this translates into a sensitivity of 88.3% (95% CI 79.7–94.3%) and a specificity of 97.7% (95% CI 87.7–99.6%) for A to detect androgen excess. Sensitivity and specificity values for T of 65.1% (95% CI 54.1–75.1%) and 88.3% (95% CI 74.9–96.1%) are less impressive. If we consider the age-related normative reference values for A in 985 women described by Haring et al (37), the demarcation between the two groups would improve the sensitivity even further, with minimum reduction of specificity.

Although serum LH values increased across each subgroup, no significant difference was observed between NA/NT women and healthy controls. We would not recommend the use of LH or the LH to FSH ratio as investigative tools in a routine PCOS workup. LH values fluctuate widely across the menstrual cycle and accurate blood sampling is inconsistently timed in the early follicular phase. It is also very unreliable in women with oligo-ovulation (29). Similarly, fasting insulin levels and HOMA-IR values were noted to increase across each androgenemic subgroup; however, these surrogate markers in isolation clearly cannot be used as a diagnostic marker for PCOS due to poor specificity, failing to accurately differentiate between PCOS-driven and multifactorial insulin resistance (7).

There have been many attempts over the last 25 years to define PCOS (1, 4, 5). Each definition focuses on hyperandrogenism and hyperandrogenemia, yet few attempts have been made to precisely define either term, and biochemical definitions remain particularly elusive. Previous work has advocated measurement of a single morning serum T sample during the early follicular phase as the diagnostic criterion for hyperandrogenemia (9). The limitations of total T as a solitary diagnostic tool for hyperandrogenemia have been described above. The clearance and bioavailability of T are affected by serum SHBG levels, which are lowered by hyperinsulinemia, but A levels are not affected by this phenomenon (38), and this may explain some of the observed discrepancies between T and A measurements. Appropriateness and reliability of analytical methods has equally been a major issue in this debate, and The Endocrine Society has appealed to international laboratories to refine techniques for androgen measurement in women (39). However, the advent of LC/MS-MS has dramatically improved the rapid detection and reliable quantification of serum steroids in both clinical and research practice (40). Accurate A measurement could





**Figure 3.** Total androgen metabolites (A), HOMA-IR (B), ISI (C), fasting insulin (D), serum DHEA (E), and DHEAS (F) in the three androgen phenotype PCOS subgroups and healthy controls. \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ .

now emerge from these developments as a sensitive diagnostic test for hyperandrogenemia in PCOS.

Patterns of body fat distribution on DXA did not differ between PCOS and control patients, supporting previously published work (41), nor were there differences in fat distribution between the androgenemic subgroups. These and other studies point toward a qualitative rather than quantitative defect in adipose biology in PCOS (42). 17 $\beta$ -HSD5 is expressed in adipose tissue, and significant extraovarian conversion to T may occur in obesity (18). 17 $\beta$ -HSD5 is also highly expressed in the ovary, and activating variants result in a PCOS phenotype with severe insulin resistance (43). This suggests a possible primary role for androgen excess in insulin resistance. However, the association of insulin resistance and hyperandrogenism could equally point toward an insulin-mediated effect on ovarian thecal steroidogenesis. The observation that insulin plays a key role in thecal androgen generation in vivo and in vitro supports this hypothesis (44, 45). Insulin-stimulated A generation from the ovarian theca may

create an enlarged circulating pool for conversion to T, and elevated levels of the former may therefore be a surrogate marker for metabolic dysfunction.

A is also produced in significant quantities by the adrenal gland, but so far, studies on the impact of hyperinsulinism on adrenal androgen production are lacking. Impaired DHEA sulfation due to 3'-phosphoadenosine 5'-phosphosulfate synthase-2 deficiency has been shown to result in a PCOS phenotype (46). A recent paper described two sulfotransferase-2A1 genetic variants that were associated with an increase in the DHEA to DHEAS ratio in a large PCOS cohort (47); in our cohort, 10% of the PCOS patients were found to have a highly elevated DHEA level but low normal serum DHEAS, indicative of reduced DHEA sulfation capacity.

One limitation of our study is its cross-sectional nature. Whether the concurrent presence of elevated A and T corresponds to an increased risk of cardiovascular disease or a worse outcome in terms of hard clinical endpoints can be ascertained only by prospective studies. Longitudinal studies will reveal whether initial increases in androgen precursors later followed by

additional increases in T may represent a sequence over time in individual patients. Although HOMA-IR measurement is a limited marker of insulin resistance, the ISI devised by Cederholm and Wibell (25) calculated in our cohort has been shown to perform with comparable accuracy to a hyperinsulinemic euglycemic clamp ( $\sim R = 0.8$ ). Similarly, it was not possible to measure serum androgens in the early follicular phase of all patients, largely due to the fact that most PCOS patients were oligo- or amenorrhoeic. Finally, the debate over what constitutes normal androgen values continues, and quoted reference ranges refer to normative values in 95% of an unselected presumptively healthy female population, implying that abnormal levels will be observed in 5% of women with no evidence of endocrinopathy.

In conclusion, these data endorse the hypothesis that increasing androgen burden is associated with an adverse metabolic phenotype and that concurrent measurement of both A and T highlights a PCOS cohort that appears to be at increased metabolic risk. We also believe on the basis of

these data that serum A is a more sensitive indicator of PCOS-related androgen excess than serum total T concentrations. The utility of this novel strategy for predicting metabolic risk in PCOS women warrants investigation in prospective and longitudinal studies and these may ultimately afford a personalized approach to the management of PCOS.

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Address all correspondence and requests for reprints to: Professor Wiebke Arlt, Centre for Endocrinology, Diabetes, and Metabolism, School of Clinical and Experimental Medicine, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom. E-mail: w.arlt@bham.ac.uk.

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